Avoiding Diagnostic Dilemmas in Routine Rabies Testing

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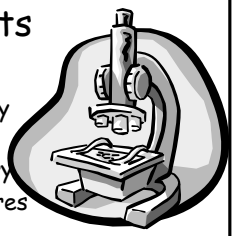
Introduction

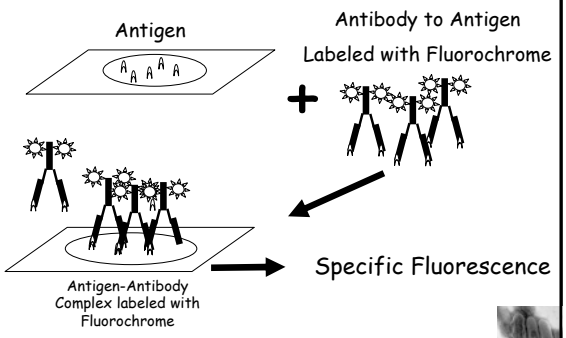
Since the development of the first rabies vaccine accurate and timely diagnosis of rabies infections in animals has been essential to prompt and successful post-exposure treatment of humans.

Diagnostic Tests



- Direct Fluorescent Antibody Test
- Rapid Immunohistochemistry
- Virus Isolation in Cell Cultures
- Virus Isolation in Mice
- Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Direct Fluorescent Antibody Test



Avoiding Diagnostic Dilemmas in Routine Rabies Testing

Involves factors such as :

1. Sample
2. Reagents
3. Technical Expertise
4. Interpretation of Results

Sample Collection

Step 1 - Avoiding Cross-Contamination

- NLTN/CDC Video- Removal of Brains for Rabies Diagnosis
- Wear appropriate PPE and maintain safety precautions as described in CDC/NIH Biosafety in Microbiological and Biomedical Laboratories (BMBL) 4 th edition
- Store clean instruments, containers, lab mats and slides in a clean area.
- Work with one specimen at a time.
- Use a clean set of instruments for each specimen.
- Label each sample immediately with a unique number
- Change gloves between samples
- Store all samples and animal carcasses until tests have been reported.
- Decontaminate the necropsy table after processing each specimen
- Decontaminate all instruments by autoclaving 121º C for 60 minutes, and clean before re-using.

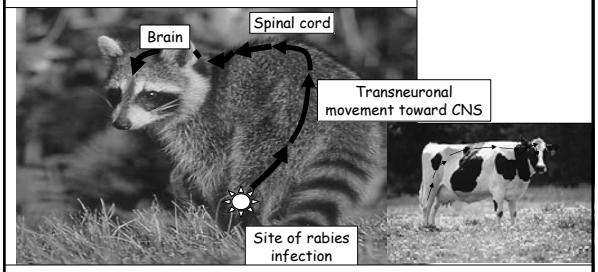
Sample Collection

Extreme care must be taken:

- To insure appropriate numbering and labeling of the sample
- To avoid cross-contamination

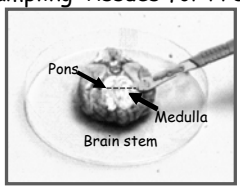
- Mistakes made at necropsy can not be easily resolved by repeat testing and performance of confirmatory tests.
- Methods such as isolation (e.g. cell culture and mouse inoculation) and RT-PCR may amplify a rabies virus contaminant.

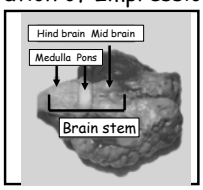
Rabies Pathogenesis –Rationale for Sample Collection

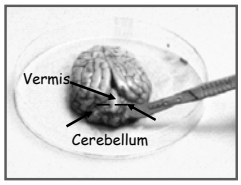


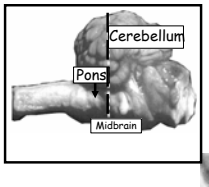
Viral antigen is widespread in most animals, but may also be unilateral especially in large animals (livestock).
Thorough examination of the brain stem and cerebellum is necessary to make a diagnosis. A cross-section through the brain stem will sample maximum ascending and desending nerve tracts.

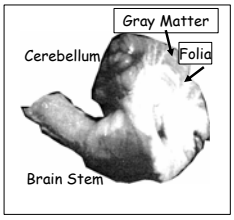
Sampling Tissues for Preparation of Impressions

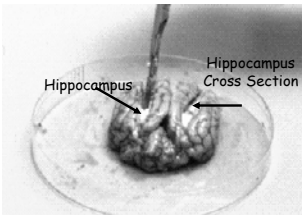












Inclusion of cerebellum or hippocampus samples gives additional confidence in making a rabies diagnosis

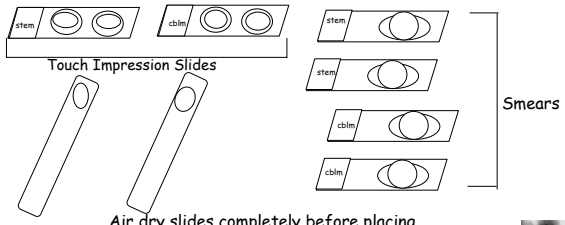
Unacceptable Samples

1. Deteriorated or decomposed samples are samples which have lost distinguishing structural characteristics, display substantial green coloration, liquefaction, desiccation. (Loss of tissue during staining and presence of bacteria may indicate decomposition.)
2. Negative results should not be reported on deteriorated samples. The test report should state that rabies can not be ruled out due to the condition of the sample.
3. Formalin-fixed tissues can not be tested by the standard DFA. Chemical cross-linking of proteins interferes with antigen binding. Other tests, FFDX DFA and IHC protocols, can be used.

Slide Preparation

Touch Impressions or Smears (duplicates of each cross section are stained with 2 different conjugates)

1. Cross section of brain stem
2. Cross section through cerebellum (vermis, right and left lateral lobes)



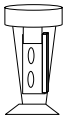
Air dry slides completely before placing acetone may take 15-30 minutes

Touch Impressions or Smears

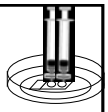
- Touch impressions or smears should be thin.
- Blotting of slides on paper towels can remove excess brain tissue.
- Thick impressions or smears may trap rabies conjugates and make interpretation difficult due to nonspecific fluorescence.
- Thick smears are more likely to be washed off in the Rinse

Acetone Fixation

1. Place each set of slides from a specimen in a separate container
2. Fix in fresh acetone at –20 C for 1 hour to overnight in an explosion proof freezer
3. Fix a set of positive and negative control slide at the same time in separate containers



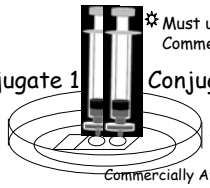
Immunofluorescent Staining



1. Samples must be tested with 2 different conjugates.
2. Conjugates are prefiltered through low protein binding 0.45 um filters attached to syringes and added directly to the slides (discard the first 3 drops of conjugate).
3. Add to the positive control first, test slides, and then last the negative control slides to insure that specific antibodies are not adsorbed by the filter in the initial drops.

- Must use 2 different conjugate preparations. Commercial conjugates are listed below.

Conjugate 1 Conjugate 2



Incubate 37°C 30 min in moist chamber

Commercially Available Conjugates

- Fujirubio Diagnostics Inc, Centocor Anti-rabies Monoclonal Globulin #800-090 (Mixture 2 IgG2a Mabs, FITC labeled)

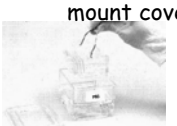
  Chemicon International Inc, Light Diagnostics Rabies DFA Reagent II #5500 (Contains the same two IgG2a Mabs as Centocor 800-090, FITC labeled)
- Chemicon, Light Diagnostics Rabies DFA Reagent #5100 (Mixture of 2 IgG1 Mabs and 1 IgG2 Mab, FITC labeled)
- Chemicon, Light Diagnostics Rabies Polyclonal DFA Reagent#5199 (Goat hyperimmune serum, FITC labeled)

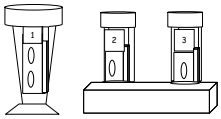
Why 2 different conjugates?

- Commercial hyper-immune (polyclonal) conjugates are broadly reactive, but may have some non-specific reactions to agents other than rabies.
- Monoclonal antibody conjugates contain 2 or 3 monoclonal antibodies to highly conserved rabies virus N-protein epitopes.
- If used at the optimal working dilution, the commercial conjugates should detect all rabies variants found in the USA.
- If there is lack of reactivity of one of the MAbs in a conjugate a reduction in reactivity will be observed.

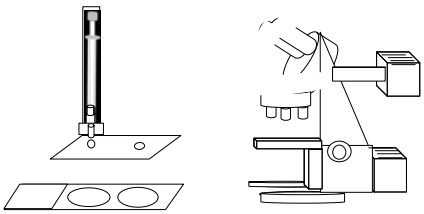
Wash PBS

1. Place each set of samples in separate container for washes (coplin jars, centrifuge tubes or staining dishes)
2. Immerse and soak in PBS for 3-5 min
3. PBS is discarded and replaced for additional 3-5 min
4. No water rinse is necessary, blot and mount coverslips.

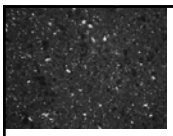




- Mount Coverslips with 20 % Glycerol Tris Buffered Mounting Medium pH 9.0



Examine slides with a Fluorescence Microscope



- Criteria for Evaluation

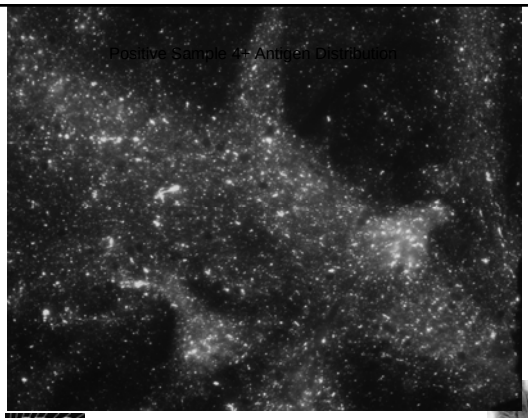
Staining intensity is graded +4 to +1

Antigen Distribution:
+ 4 Massive infiltration of small and large antigen in almost every area of slide

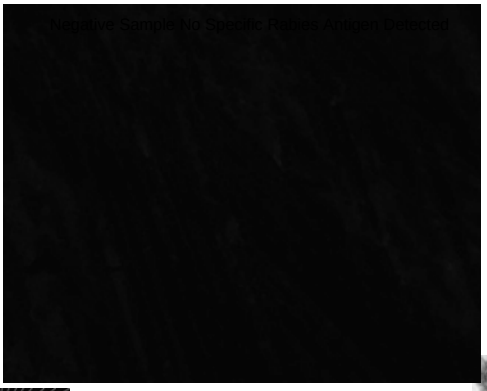
+3 inclusions of varying size and shape in every field, number of inclusions vary, but are numerous

+2 inclusions of varying size and shape in 10-50% of fields and most field contain only a few inclusions

+1 inclusion of various size and shape are present in <10% of the microscopic fields and only a few inclusions are found per field



Positive Sample 4+ Antigen Distribution



Negative Sample No Specific Rabies Antigen Detected

Interpretation of Results

- Complete Tests

If the positive and negative control slides give the appropriate results, the sample tissues were in satisfactory condition, and adequate amounts of tissue were tested, then tests can be considered complete or incomplete based on observed patterns of staining.

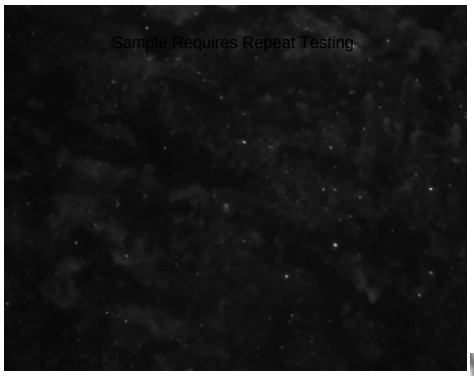
Negative – No specific staining in the test slides with 2 different anti-rabies conjugates

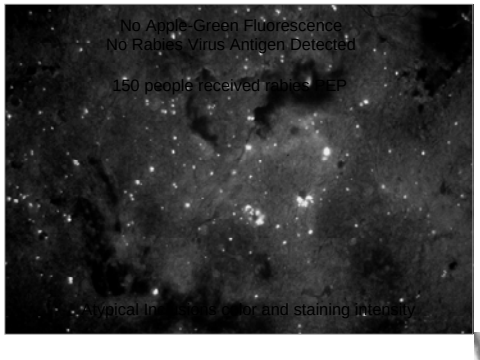
Positive- Clearly positive with both anti-rabies conjugates (+3-4 staining and +2-+4 antigen distribution)

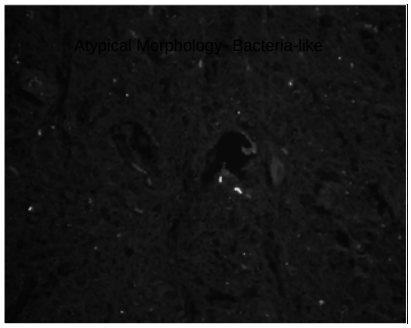
Interpretation of Results

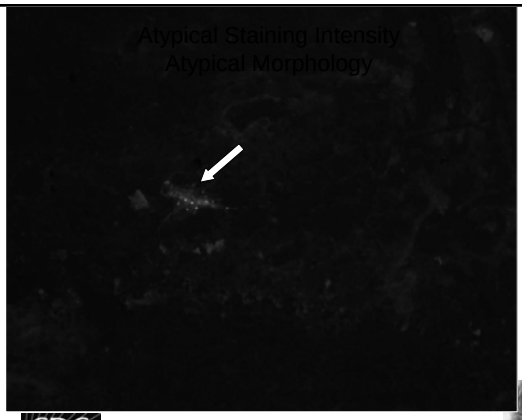
- Incomplete Tests

Typical Inclusions	< 10% of examined fields.
	> 10% with sparse distribution 1-2 per field.
	weak intensity < +3
Atypical	inclusion morphology but with +4 intensity, regularly sized uniformed texture
	Fluorescing bacteria; might mask small amounts of rabies specific staining
	Particulate or free fluorescein; might mask small amounts of rabies specific staining
Discordant results	with 2 reagents or 2 readers







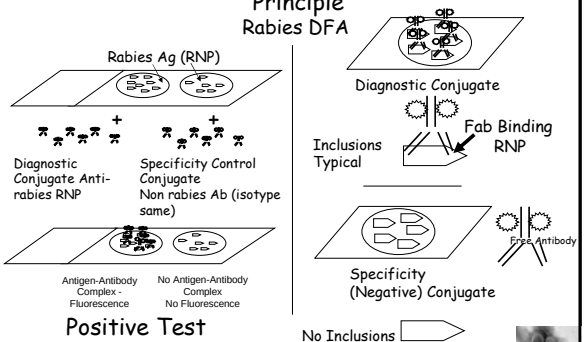


☼ Repeat Testing/Confirmatory Test

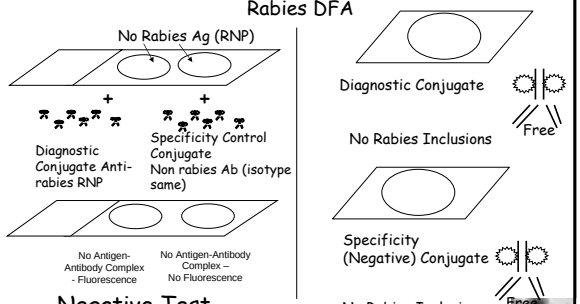
All incomplete DFA tests must be repeated with 2 conjugates and at least one specificity control reagent (negative control conjugate).

Slides are remade from the original brain tissues in the same manner as previously described. (At least 3 impressions or smears from each tissue are required.)

☼ Specificity Controls

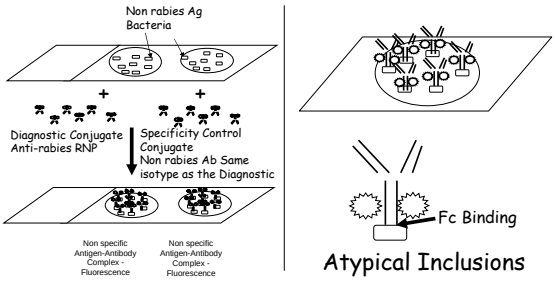


Specificity Controls Principle
Rabies DFA



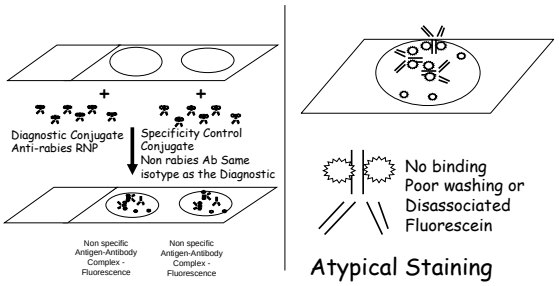
Negative Test

Nonspecific Fluorescence



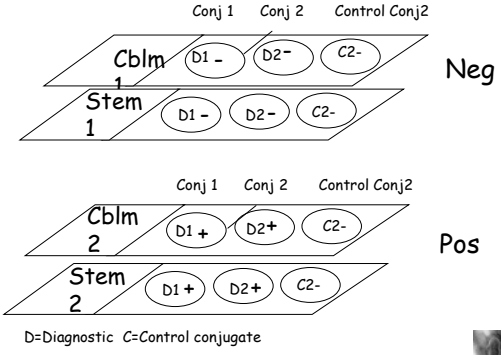
Atypical Inclusions

Nonspecific Fluorescence



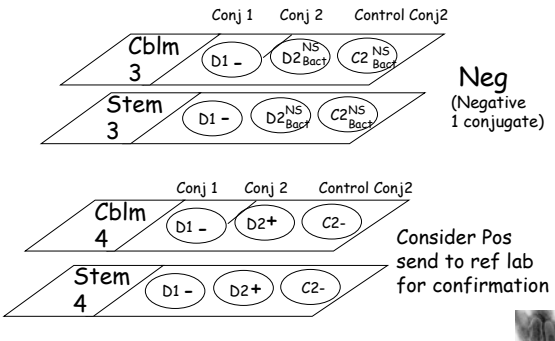
Atypical Staining

Repeat DFA



D=Diagnostic C=Control conjugate

Repeat DFA



New Standard Protocol Requires:

1. Adequate sampling of brain tissues from the brain stem (cross section through pons or medulla) and from cerebellum (vermis, right and left lobes)
2. Acetone fixation at –20 C for 1 hour to overnight in a separate container for each sample's set of slides
3. Use of 2 anti-rabies conjugates in every test
4. Rinse slides in 2 changes of PBS for 3-5 minutes; separate containers are required for each set of slides
5. Use of 20 % glycerol Tris buffered mountant
6. Two readers to evaluate the test slides
7. Repeat testing of weakly positive and inconclusive tests using a rabies conjugate and specificity (negative) control conjugate

Conclusions

- The Protocol for Post-mortem Diagnosis of Rabies in Animals- Minimum Standard for the United States includes: steps to avoid cross-contamination, maintain sensitivity and specificity, criteria for evaluation of test results, confirmatory testing repeat DFA testing and submission to a reference laboratory.
- Compliance of US laboratories to these standards is essential.
- Pre-testing procedures to avoid cross-contamination during brain removal and preparation of impression smears are not specifically addressed in the protocol. Although these issues were addressed in the CDC videotape: *Removal of Animal Brains for Rabies Diagnosis*
- Additional training materials (slides or atlas) need to be developed to familiarize the laboratorian with color, intensity and morphology of specific rabies inclusions.

Conclusions

- Alternate confirmatory tests need to be investigated. Direct rabies immunohistochemical test (DRIT) seems to be a highly sensitive test for rabies virus antigen detection and a possible confirmatory test.
- RT-PCR is a sensitive method, however, limitations include condition of sample and RNA, primer match, protocol used. Until true universal primers are developed to amplify all rabies virus variants the usefulness of RT-PCR as a diagnostic tool will be limited.
- The National Working Group on Rabies Diagnosis should meet to address some of these issues as well as discuss problems associated with compliance.

Compliance

- Although 115 of the 121 laboratories performing rabies diagnosis surveyed in 2003 were familiar with the standard protocol only 45/115 (39%) performed the minimum standard protocol as written.
- NLTN/CDC Rabies training courses were held in Jan and March 2004 and Jan 2006 to acquaint laboratorians with the standardized protocol

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